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Glial Dysfunction Causes Age-Related Memory Impairment in *Drosophila*

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SUMMARY

Several aging phenotypes, including age-related memory impairment (AMI), are thought to be caused by cumulative oxidative damage. In *Drosophila*, age-related impairments in 1 hr memory can be suppressed by reducing activity of protein kinase A (PKA). However, the mechanism for this effect has been unclear. Here we show that decreasing PKA suppresses AMI by reducing activity of pyruvate carboxylase (PC), a glial metabolic enzyme whose amounts increase upon aging. Increased PC activity causes AMI through a mechanism independent of oxidative damage. Instead, increased PC activity is associated with decreases in D-serine, a glia-derived neuromodulator that regulates NMDA receptor activity. D-serine feeding suppresses both AMI and memory impairment caused by glial overexpression of dPC, indicating that an oxidative stress-independent dysregulation of glial modulation of neuronal activity contributes to AMI in *Drosophila*.

INTRODUCTION

Various mechanisms have been proposed to cause age-related memory impairment (AMI), including increases in mitochondrial dysfunction, decreases in insulin/IGF signaling, decreases in autophagy and protein turnover, and alterations in expression of genes involved in neuronal function and stress-response pathways (Bishop et al., 2010; Gupta et al., 2013). Most of these mechanisms are thought to occur as a consequence of an initial age-dependent increase or accumulation of oxidative damage. However, while methods of reducing oxidative damage have been reported to increase lifespan (Sampayo et al., 2003; Sun and Tower, 1999), the role of oxidative damage as a cause of AMI is still controversial (Hirano et al., 2012; Liu et al., 2003).

A number of genomic and proteomic analyses in various organisms have identified age-related changes in expression of many transcripts and proteins (Jiang et al., 2001, 2010; Kim et al., 2005; Lu et al., 2004; Sato et al., 2005). However, the identification of pathways and genes causing AMI has been hindered by the difficulty of distinguishing age-associated changes in general from those specific to AMI. In *Drosophila*, AMI consists of defects in transcription-independent middle-term memory (MTM), which can be measured 1 hr after training (Horiuchi and Saitoe, 2005; Tamura et al., 2003; Tonoki and Davis, 2012), and in transcription-dependent long-term memory (LTM), often measured 24 hr after multiple spaced trainings (Mery, 2007). Previously, we showed that age-related impairments in 1 hr memory are suppressed by heterozygous mutations in the *DC0* (*DC0*/+) gene, which encodes the major catalytic subunit of cAMP-dependent protein kinase (PKA) (Yamazaki et al., 2007). Furthermore, decreasing PKA activity in the mushroom bodies (MBs), neural centers essential for forming associative olfactory memories, at old ages reverses this impairment, while increasing PKA activity in the MBs at young ages causes premature impairment (Yamazaki et al., 2010). In mammals, an age-dependent decline in prefrontal cortex-dependent working memory is ameliorated by infusing PKA inhibitors and enhanced by infusing a PKA activator, demonstrating that PKA's role in AMI may be conserved (Ramos et al., 2003). However, amounts of cAMP and activity of PKA do not increase upon aging, suggesting that there are likely to be other genes that show age-related changes in activity and cause AMI through a PKA-dependent mechanism.

DC0/+ mutations suppress AMI without changing lifespan (Yamazaki et al., 2007). Therefore, we reasoned that AMI-associated proteins might be found by identifying differences between age-related changes in protein expression in wild-type flies and *DC0*/+ mutants. Using proteome analysis, we identified the

Drosophila homolog of pyruvate carboxylase (dPC) as a protein whose expression is increased upon aging and is decreased in *DC0*/+ mutants. PC is a mitochondrial anaplerotic enzyme that carboxylates pyruvate to form oxaloacetate (OAA) and is expressed in glial cells in the mammalian brain (Hertz et al., 2007; Jitrapakdee et al., 2006; Shank et al., 1985). Although lack of PC activity causes lactic acidosis, which leads to mental retardation and early death during childhood in humans (García-Cazorla et al., 2006; Israels et al., 1976), our current study demonstrates that an age-related increase in dPC activity in glial cells is a cause of AMI in *Drosophila*. This increase does not occur as a response to oxidative damage, suggesting that an alternative pathway causing dysregulation of glia modulation of neuronal activity may be a cause of AMI.

RESULTS

Identification of AMI-Associated Protein Changes

Age-related impairments in 1 hr memory occur within 20 days of age in wild-type but not in *Pka-C1* heterozygous (DCO^{B3}/+) flies (Yamazaki et al., 2007). Thus, to identify potential AMI-associated proteins, we searched for proteins that showed altered expression upon aging to 20 days in a wild-type background and a shift toward young expression in 20-day-old DCO^{B3}/+ flies compared to 20-day-old wild-type flies. We performed this screen using fluorescence-based two-dimensional differential gel electrophoresis (2D-DIGE) (Jiang et al., 2010).

We screened for protein spots that showed a more than 1.5-fold change in spot intensity and found 91 differences between young and aged wild-type flies (n = 3, Figure S1A available online), 94 differences between young and aged DCO^{B3}/+ flies (n = 3, Figure S1B), and 9 differences between aged wild-type and aged DCO^{B3}/+ flies (n = 3, Figure S1C). Among these differences, one cluster of three spots (open arrowheads in Figures S1A–S1C) consistently showed intensities that increased upon aging but were lower in 20-day-old DCO^{B3}/+ flies compared to 20-day-old wild-type flies. Using mass spectroscopy, we determined that all spots in this cluster are derived from the *CG1516* gene (http://flybase.org/reports/FBgn0027580.html).

Heterozygous Mutations in *CG1516* Suppress AMI

The principal phenotype of AMI in wild-type flies after single-cycle olfactory conditioning is a specific reduction in middle-term memory (MTM), characteristically measured 1 hr after single-cycle olfactory conditioning (Tamura et al., 2003; Tonoki and Davis, 2012). To determine whether *CG1516* is associated with AMI, we assayed 1 hr memory in young and old *CG1516* mutants after single-cycle training.

We identified two fly lines, *EP2547* and *CB0292-3*, which have P element transposon insertions in *CG1516* (Figure 1A). Homozygous *EP2547* and *CB0292-3* flies are semilethal and express significantly reduced amounts of CG1516 product (Figure 1B). They also have severe reductions in 1 hr memory but normal task-related sensorimotor responses (Figures S2A–S2C). Heterozygous *EP2547*/+ and *CB0292-3*/+ flies are viable, express reduced amounts of CG1516 product (Figure 1B), and have normal or near normal 1 hr memory at young ages. Significantly, both heterozygous lines showed suppression of AMI compared to wild-type flies (Figures 1C and 1D), with no extension of lifespan (Figures S2D and S2E). In addition, memory retention curves of aged *EP2547*/+ and *CB0292-3*/+ flies were not significantly different from young flies (Figures 1E and 1F).

CG1516 Encodes *Drosophila* Pyruvate Carboxylase

Based on sequence similarity, *CG1516* is predicted to encode *Drosophila* pyruvate carboxylase (dPC) (Figure 2A). In humans, PC deficiency is associated with severe mental retardation (García-Cazorla et al., 2006; Israels et al., 1976), a phenotype consistent with what we observed in homozygous *EP2547* and *CB0292-3* flies (Figure S2A). PC catalyzes the ATP-dependent carboxylation of pyruvate to oxaloacetic acid (OAA) in mitochondria and is exclusively expressed in glial cells in the mammalian brain (Hertz et al., 2007; Jitrapakdee et al., 2006; Shank et al., 1985). OAA is further converted to malate by malate dehydrogenase (MDH) in a reaction that reduces NADH (Modak and Kelly, 1995). Thus, to test whether *CG1516* encodes dPC, we expressed a C-terminal myc-tagged *CG1516* transgene in glial cells of flies, using a glial-specific *repo-GAL4* driver (Xiong et al., 1994) and measured the ability of myc-antibody immunoprecipitates to reduce NADH in the presence of MDH (Modak and Kelly, 1995). Anti-myc antibody immunoprecipitates exhibited robust NADH reduction activity in a complete assay mixture (see Experimental Procedures for details), whereas immunoprecipitates using control anti-tubulin antibody did not (Figure 2B). NADH was not reduced by anti-myc immunoprecipitates in the absence of the substrate pyruvate or ATP. Moreover, NADH reduction was also greatly inhibited in the absence of $NaHCO_3$ (a source of CO_2). These results indicate that *CG1516* probably encodes dPC, and we confirmed that amounts of *CG1516* gene product, hereafter referred to as dPC, increases significantly upon aging (Figure 2C). We further demonstrated that heterozygous dPC^{EP2547}/+ and $dPC^{CB0292\text{-}3}$/+ mutations reduce dPC activity (Figure 2D).

Glial dPC Activity Is Responsible for AMI

Mammalian PC is expressed in astrocytes but not in neurons (Hertz et al., 2007; Shank et al., 1985). To determine whether dPC is also located in glia, we used a mitochondrially targeted GFP construct, *mito-GFP* (Pilling et al., 2006), expressed from a glial-specific *repo-GAL4* driver, to visualize glial mitochondria. As shown in Figure 3A, GFP signals are observed in a punctate pattern surrounding neuropil regions. We observed an overlapping pattern when we probed with anti-dPC antibodies (Figures 3B and 3C). Although we also observed some dPC signals in neuropil regions including the calyx, these signals remained unchanged in a $dPC^{EP2547}/dPC^{CB0292\text{-}3}$ background, suggesting that they are nonspecific (Figures S3A, S3B, and S3C). In contrast, glial signals were abolished in a $dPC^{EP2547}/dPC^{CB0292\text{-}3}$ background. We further demonstrated that $dPC^{MI02451}$ flies, which carry a MiMIC transposon (a Minos-mediated integration cassette including a GFP transgene) (Venken et al., 2011) in the *dPC* locus, also show GFP signals predominantly in glia (Figures S3D, S3E, and S3F).

To test whether glial *dPC* is responsible for AMI, we expressed a *dPC* transgene (dPC^{+}) using two glial drivers,

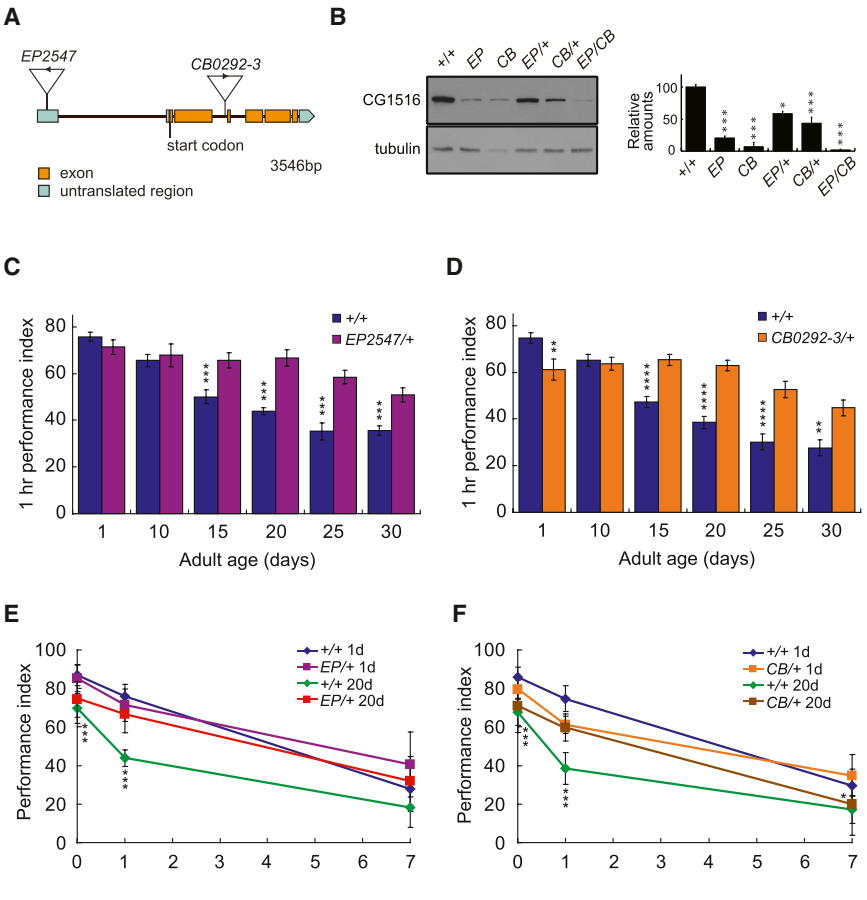

Figure 1. Heterozygous Mutations in CG1516 Suppress AMI

(A) A schematic diagram of the *CG1516* gene and *EP2547* and *CB0292-3* P element insertions.

(B) The protein encoded by *CG1516* is significantly reduced in *EP2547*, *CB0292-3*, *EP2547*/+, *CB0292-3*/+, and transheterozygous *EP2547*/*CB0292-3* flies as assayed by western blots. Amounts of protein, normalized to tubulin, are reduced to 20.9% ± 4.0%, 7.1% ± 6.6%, 60.0% ± 2.5%, 43.3% ± 8.0%, and 1.8% ± 1.2% of wild-type amounts, respectively. One-way ANOVA reveals significant differences between genotypes ($F_{5,12}$ = 67.04, p < 0.0001). *p < 0.05, ***p < 0.001 compared to wild-type. n = 3.

(C and D) AMI is suppressed in *EP2547*/+ (C) and *CB0292-3*/+ (D) mutants. Two-way ANOVA revealed significant differences due to age ($F_{5,103}$ = 75.07, p < 0.0001 for *EP2547*/+ and $F_{5,100}$ = 41.52, p < 0.0001 for *CB0292-3*/+), genotype ($F_{1,103}$ = 10.15, p < 0.0001 for *EP2547*/+ and $F_{1,100}$ = 51.79, p < 0.0001 for *CB0292-3*/+), and interaction between age and genotype ($F_{5,103}$ = 33.37, p < 0.0001 for *EP2547*/+ and $F_{5,100}$ = 15.44, p < 0.0001 for *CB0292-3*/+). Bonferroni post hoc analysis demonstrated significant differences in 1 hr memory between wild-type and mutants at 15 days of age and older. **p < 0.01, ***p < 0.001, ****p < 0.0001.

(E and F) Memory retention curves of wild-type, *EP2547*/+ (E), and *CB0292-3*/+ (F) flies at 1 day and 20 days of age. Two-way ANOVA reveals significant differences due to genotype ($F_{3,107}$ = 30.06, p < 0.0001 for E; $F_{3,99}$ = 26.07, p < 0.0001 for F), retention time ($F_{2,107}$ = 278.2, p < 0.0001 for E; $F_{2,99}$ = 256.2, p < 0.0001 for F), and interaction between genotype and retention time ($F_{6,107}$ = 3.174, p = 0.0066 for E; $F_{6,99}$ = 4.214, p = 0.0008 for F). Bonferroni post hoc analysis demonstrated significant differences due to aging in 0 hr and 1 hr memory in wild-type flies and in 7 hr memory in *CB0292-3*/+ flies. *p < 0.05, ***p < 0.001. n = 7–12 for data in (C)–(F). Data in all bar and line graphs of this paper represent means ± SEMs.

repo-GAL4 and *nrv2-GAL4*, and found that AMI was restored in a dPC^{EP2547}/+ background (Figure 3D). In contrast, neuronal expression of dPC^{+} from an *elav-GAL4* driver had no effect on AMI. Using an RU486-inducible glial geneswitch driver, we further demonstrated that glial dPC activity at the adult stage, but not the larval stage, restores AMI in this background (Figure 3E). Moreover, 3 day feeding of RU486 specifically to old *GliaGS* > *UAS-dPC*$^{+}$;dPC^{EP2547}/+ flies restores AMI (Figure 3E), suggesting that acute increases in dPC are sufficient to induce AMI.

If increased glial dPC activity causes AMI, overexpressing dPC^{+} in glia may prematurely disrupt 1 hr memory in young flies. Young *repo-GAL4>UAS-dPC*$^{+}$ and *nrv2-GAL4>UAS-dPC*$^{+}$ flies had severely reduced 1 hr memory compared with young wild-type flies (Figure 3F). In contrast, overexpressing dPC^{+} either panneuronally or in the MBs did not affect memory. Neither initial learning, which was assayed immediately after training, nor 7 hr memory were affected in either *repo-GAL4* > *UAS-dPC*$^{+}$ or *nrv2-GAL4>UAS-dPC*$^{+}$ flies (Figures S3G and S3H), suggesting that increasing dPC activity in glial cells specifically disrupts the memory phase affected in AMI. Consistent with this idea, aging did not further exacerbate the reduction in 1 hr memory observed in *repo-GAL4>UAS-dPC*$^{+}$ and *nrv2-GAL4>UAS-dPC*$^{+}$ flies, indicating that increasing amounts of glial dPC functions to occlude AMI (Figure S3I).

Glial dPC Activity Mediates PKA's Effects on AMI

Overexpression of $DC0^{+}$ in the MBs causes a premature reduction in 1 hr memory (Yamazaki et al., 2007). We found that this reduction does not occur in a *dPC* heterozygous background (Figure 4A). Further, AMI is restored in *DC0*/+ flies by overexpressing dPC^{+} in glia (Figure 4B). In combination with our 2D data, these results indicate that neuronal PKA affects AMI by regulating amounts of glial dPC. Interestingly, although the overexpression of dPC^{+} in glia in a wild-type background disrupts memory at a young age (Figure 3F), this disruption is not observed in young flies from a $DC0^{B3}$/+ background (Figure 4B), suggesting that the reductions in endogenous dPC in $DC0^{B3}$/+ flies can suppress the deleterious effects of glial dPC^{+} overexpression. Consistent with this idea, we found an approximately 2-fold decrease in dPC amounts in *DC0*/+ flies (Figure 4C). While dPC activity increases similarly upon aging in both wild-type and $DC0^{B3}$/+ flies (an ~2.5-fold increase, Figure 4D), it is reduced equally (>2-fold) in both young and old $DC0^{B3}$/+ flies, suggesting that reduced PKA suppresses AMI by decreasing dPC activity in an age-independent manner.

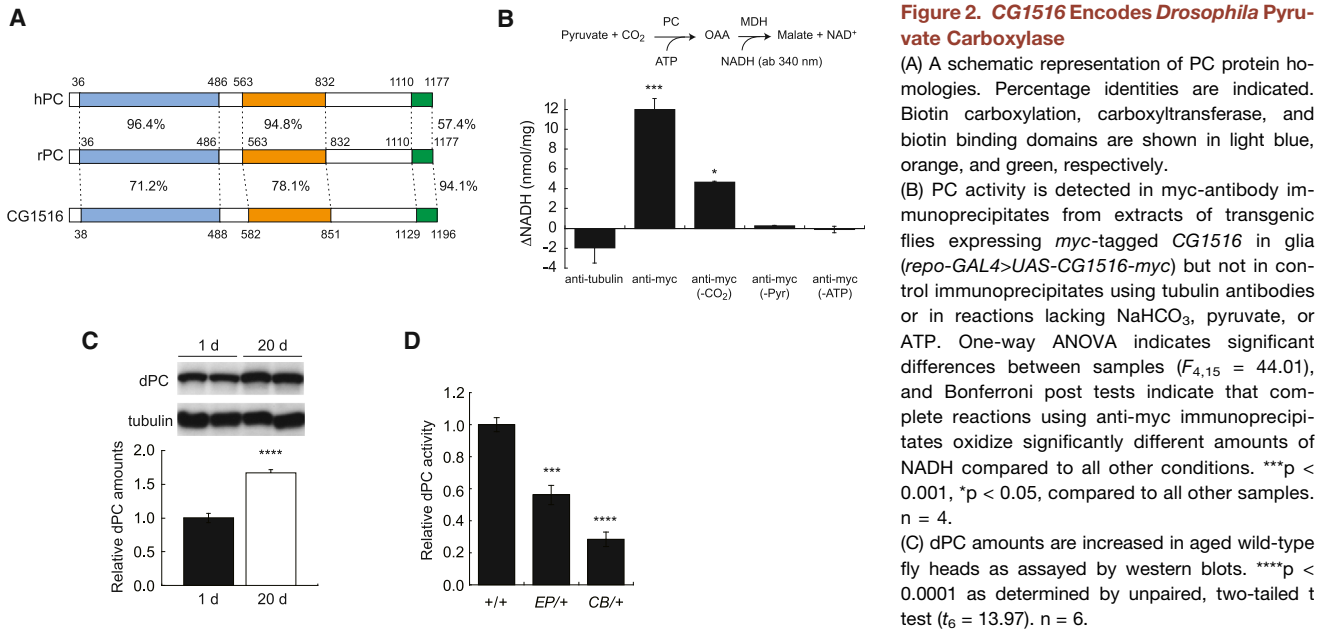

Figure 2. *CG1516* Encodes *Drosophila* Pyruvate Carboxylase

(A) A schematic representation of PC protein homologies. Percentage identities are indicated. Biotin carboxylation, carboxyltransferase, and biotin binding domains are shown in light blue, orange, and green, respectively.

(B) PC activity is detected in myc-antibody immunoprecipitates from extracts of transgenic flies expressing *myc*-tagged *CG1516* in glia (*repo-GAL4>UAS-CG1516-myc*) but not in control immunoprecipitates using tubulin antibodies or in reactions lacking $NaHCO_3$, pyruvate, or ATP. One-way ANOVA indicates significant differences between samples ($F_{4,15}$ = 44.01), and Bonferroni post tests indicate that complete reactions using anti-myc immunoprecipitates oxidize significantly different amounts of NADH compared to all other conditions. ***p < 0.001, *p < 0.05, compared to all other samples. n = 4.

(C) dPC amounts are increased in aged wild-type fly heads as assayed by western blots. ****p < 0.0001 as determined by unpaired, two-tailed t test (t_6 = 13.97). n = 6.

(D) Endogenous mitochondrial dPC activity is significantly reduced in dPC^{EP2547}/+ and $dPC^{CB0292\text{-}3}$/+ flies. One-way ANOVA reveals significant differences between genotypes ($F_{2,9}$ = 65.64). ***p < 0.001, ****p < 0.0001 compared to wild-type flies. n = 4.

dPC Amounts Do Not Correlate with Oxidative Stress

Although it is unclear whether suppression of AMI by *DC0* mutations involves the oxidative stress pathway, cumulative damage from oxidative stress is proposed to be a central cause of AMI (Bishop et al., 2010; Fukui et al., 2001; Lee et al., 2012; Levin et al., 2002). This raises the possibility that increases in dPC amounts could be caused by increased age-associated oxidative damage. However, we found that feeding flies the reactive oxygen species (ROS)-generating agent paraquat (PQ) at a concentration sufficient to kill ~50% of flies and increase expression of the oxidative stress marker, *heat-shock protein 22* (*hsp22*) >100-fold in fly heads (King and Tower, 1999; Landis et al., 2004) (Figure S4A), had no effect on dPC amounts (Figure S4B). A previous report has shown that brain functions may be protected from oxidative damage by a defense system against ROSs (Hirano et al., 2012). Thus, to study the relationship between ROSs, AMI, and dPC further, we artificially increased damage from oxidative stress by weakening this defense system using *nrv2-Gal4>UAS-sod1-IR* flies, in which *sod1*, the gene encoding the antioxidant enzyme superoxide dismutase 1, is knocked down in glia. Although *nrv2-Gal4>UAS-sod1-IR* flies have normal 1 hr memory immediately after eclosion (1 day old), memory is disrupted at 5 days of age (Figure 5A), with a concomitant increase in *hsp22* expression (Figure 5B). However, dPC amounts are unaltered (Figure 5C), and the dPC^{EP2547}/+ mutation does not ameliorate disrupted memory in these flies (Figure 5D). Knocking down *sod1* in neurons using *elav-Gal4>UAS-sod1-IR* flies similarly disrupted 1 hr memory in 10-day-old, but not 1-day-old, flies (Figure S4C). Again, however, amounts of dPC protein are unaltered upon neuronal knockdown of *sod1* (Figure S4D), and the dPC^{EP2547}/+ mutation does not ameliorate disrupted memory in these flies (Figure S4E). These results suggest that AMI and memory loss due to increasing oxidative damage occur through distinct mechanisms. Supporting this idea, the memory defects caused by overexpression of dPC^{+} in glia (Figure 3F) are not associated with increased *hsp22* expression (Figure 5E). Further, *nrv2-Gal4 > UAS-*dPC^{+} flies have normal anesthesia-resistant memory (ARM), similar to aged wild-type flies (Tamura et al., 2003), while *nrv2>sod1-IR* flies have defective ARM (Figure 5F), indicating that increasing oxidative damage affects a broader range of memory phases compared to AMI.

dPC Mutations Suppress an Age-Related Decrease of D-serine

If increased dPC activity and AMI are not associated with increased oxidative damage, how does an increase in dPC activity disrupt memory? PC activity produces OAA, which can be converted to aspartic acid (Asp) by mitochondrial aspartate transaminase (m-AST). Both OAA and Asp are potent competitive inhibitors of serine racemase (SR) (Dunlop and Neidle, 2005; Strísovský et al., 2005), which converts L-serine to D-serine. Since D-serine functions as a coactivator of NMDA receptors (NMDARs) (Henneberger et al., 2010; Martineau et al., 2006; Panatier et al., 2006), we hypothesized that reduced D-serine production may be a cause of AMI and that *dPC* mutations may suppress this reduction. To test this, we measured the ratio of D-serine to L-serine (D/L ratio) in fly brain extracts and determined that this ratio decreased significantly upon aging in wild-type flies, while it remained high in both aged dPC^{EP2547}/+ and $DC0^{B3}$/+ flies (Figure 6A). In contrast, glial overexpression of a dPC^{+} transgene (Figure 6B) and neuronal overexpression of $DC0^{+}$ (Figure 6A), caused a decrease in the D/L ratio in young flies. Thus, the D/L ratio is high in young flies and in old flies with

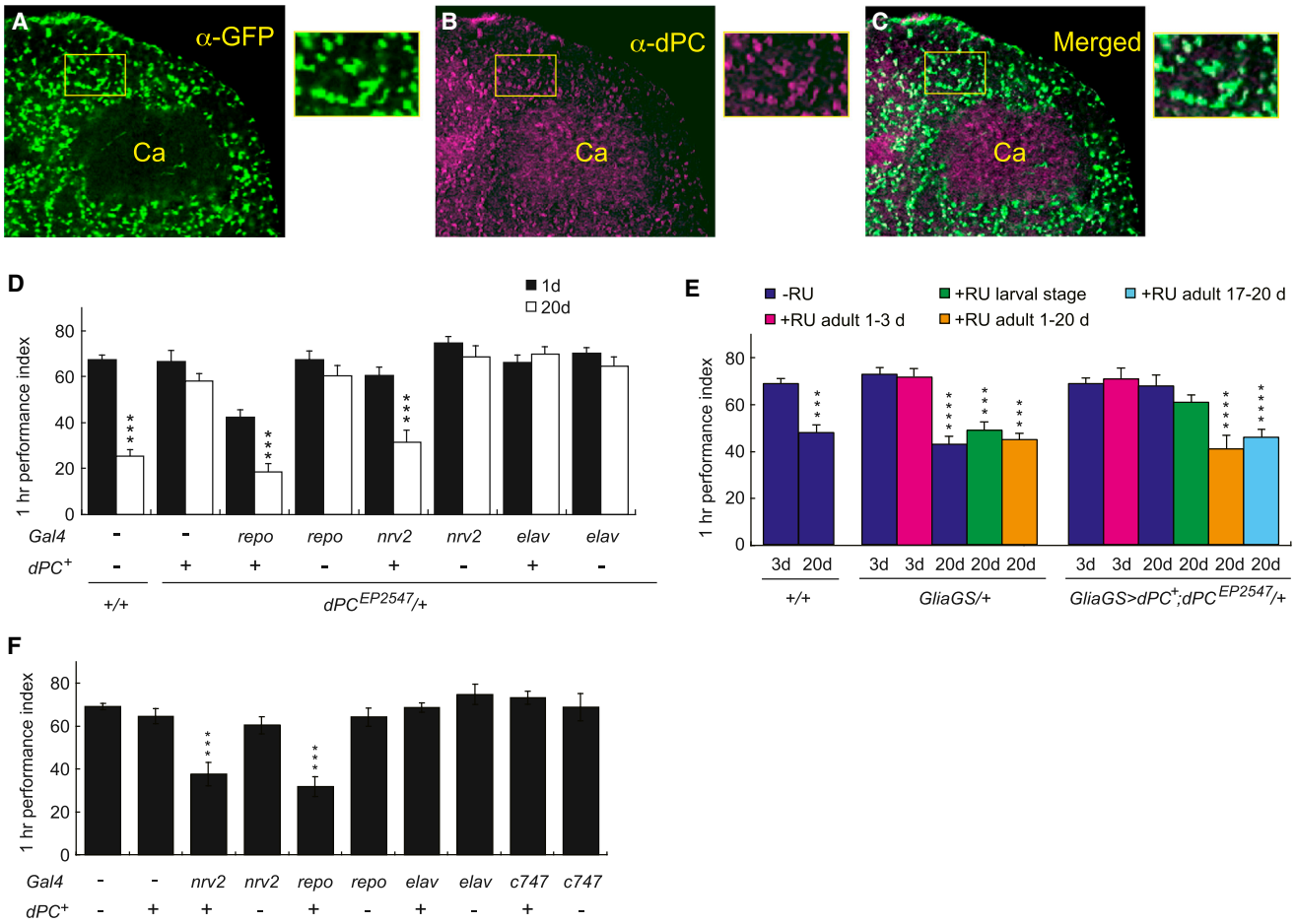

Figure 3. Glial dPC Activity Is Associated with AMI

(A–C) Colocalization (C) of mito-GFP expressed from a glial *repo-GAL4* driver (A, green) and dPC (B, red) in a representative *repo-GAL4>UAS-mito-GFP* brain. Images show an optical slice of the left dorsal brain surrounding the MB calyx (Ca). Higher-magnification images of the areas boxed in yellow are shown to the left of each panel.

(D) AMI is restored in dPC^{EP2547}/+ mutants by expressing a dPC^{+} transgene in glia (*repo* and *nrv2*) but not in neurons (*elav*). Two-way ANOVA indicates significant differences due to genotype ($F_{7,127} = 30.31$, $p < 0.0001$), age ($F_{1,127} = 62.96$, $p < 0.0001$), and interaction between genotype and age ($F_{7,127} = 8.973$, $p < 0.0001$). ***$p < 0.001$ compared to corresponding 1-day-old flies.

(E) AMI is restored in GliaGS > dPC^{+};dPC^{EP2547}/+ (*GSG3285-1* > *UAS-*dPC^{+};dPC^{EP2547}/+) flies when a dPC^{+} transgene is expressed in glia acutely during adult stages but not during larval stages. Flies were fed 1 mM RU486 for indicated times and memory was assayed in either 3-day-old (3d) or 20-day-old (20d) flies. One-way ANOVA indicates significant differences between samples ($F_{12,91} = 12.33$, $p < 0.0001$). ***$p < 0.001$ and ****$p < 0.0001$, compared to 3-day-old control *GliaGS*/+ flies fed normal food (−RU).

(F) Overexpressing dPC^{+} in glia, but not neurons nor MB (*c747*), disrupts 1 hr memory in young flies. One-way ANOVA indicates significant differences between samples ($F_{9,77} = 22.38$, $p < 0.0001$). ***$p < 0.001$ compared to all other genotypes. n = 8–12 for behavioral data.

ameliorated AMI, while it is low in old flies and in young flies with accelerated AMI. Significantly, both AMI and the memory defect caused by glial overexpression of dPC^{+} were ameliorated by feeding wild-type flies 1 mM D-serine (Figures 6C, 6D, and S5). These results suggest that an age-related increase in dPC activity causes an age-related decrease in D-serine production, leading to decreased NMDAR activity and AMI.

DISCUSSION

Several types of AMI, including decreases in 1 hr memory in *Drosophila* and working memory in mammals, can be ameliorated by treatments that inhibit cAMP/PKA signaling (Ramos et al., 2003; Yamazaki et al., 2007). We used this phenotype to identify potential AMI-associated factors downstream of PKA. Similar to other groups (Jiang et al., 2001, 2010; Kadish et al., 2009; Kim et al., 2005; Sato et al., 2005), we identified a large number of age-related changes in expression profiles in both wild-type and *DC0*/+ flies. Among these, dPC had both increased expression upon aging and decreased expression in *DC0*/+ flies compared to wild-type. In our screen, we analyzed ~2,300 protein spots, suggesting that larger-scale proteome analyses may yield additional AMI-associated proteins. On the other hand, decreasing dPC activity is sufficient to delay AMI,

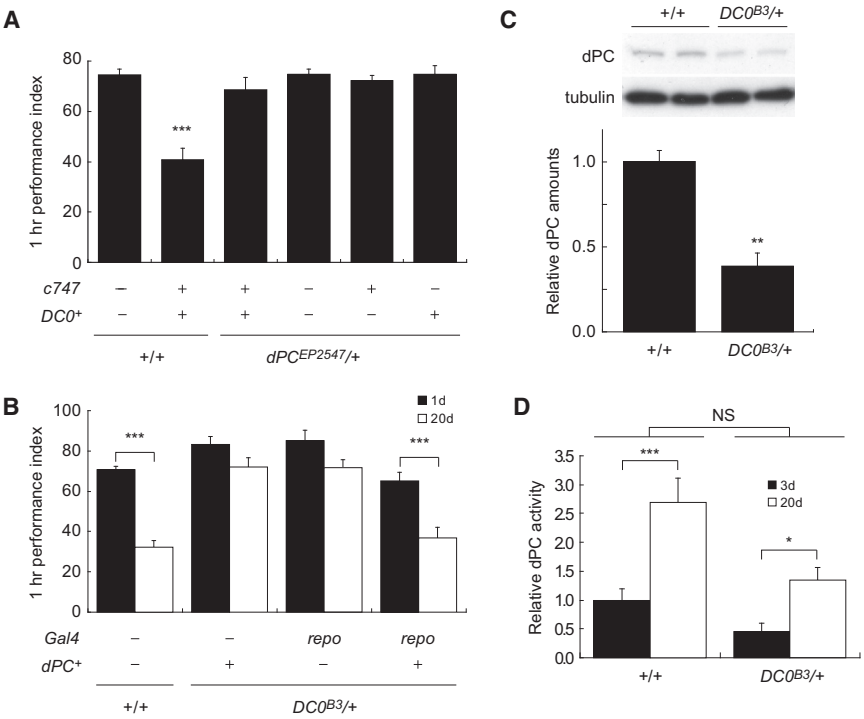

Figure 4. *DC0* Mutations Suppress AMI by Reducing dPC Activity

(A) The dPC^{EP2547}/+ mutation suppresses 1 hr memory impairment caused by overexpressing $DC0^{+}$ in the MBs. One-way ANOVA indicates significant differences between samples ($F_{5,55}$ = 11.47, p < 0.0001). ***p < 0.001 compared to all other genotypes.

(B) Overexpression of dPC^{+} in glial cells restores AMI in a $DC0^{B3}$/+ mutant. Two-way ANOVA reveals significant differences due to age ($F_{1,66}$ = 67.08, p < 0.0001), genotype ($F_{3,66}$ = 31.24, p < 0.0001), and the interaction between age and genotype ($F_{3,66}$ = 6.814, p < 0.0005). ***p < 0.001.

(C) $DC0^{B3}$/+ flies have decreased amounts of dPC. **p = 0.0047, determined by unpaired, two-tailed t test (t_8 = 3.869).

(D) Age-associated changes in endogenous dPC activity in wild-type and $DC0^{B3}$/+ flies. Two-way ANOVA of the data indicates significant differences due to genotype ($F_{1,17}$ = 21.54, p = 0.0002) and age ($F_{1,17}$ = 43.85, p < 0.0001) but not from interaction between age and genotype ($F_{1,17}$ = 4.291, p = 0.0538), indicating that the age-related increase in dPC activity is similar in both genotypes. ***p < 0.001, *p < 0.05. n = 8–12 for all behavioral data and n = 4–7 for all biochemical data.

and increasing dPC specifically inhibits the memory phase affected by AMI, suggesting that dPC is a major regulator of AMI. We note here that overall AMI in *Drosophila* consists of decreases in both 1 hr memory and long-term memory. In this study, we focus on mechanisms involved in decreases in 1 hr memory after single-cycle training. *DC0*/+ and *dPC*/+ mutants do not affect age-dependent impairments in long-term memory after multiple spaced trainings (data not shown).

While PKA activity in the mushroom bodies affects AMI (Yamazaki et al., 2010), dPC is found predominantly in glia, and glial dPC activity is associated with AMI. Thus, although our 2D-DIGE data show three protein spots for dPC, indicative of different phosphorylation states, we do not think that dPC is a direct substrate of PKA. Our genetic and biochemical data indicate that glial dPC amounts are regulated by neuronal PKA, and that PKA's effects on AMI depend on glial dPC activity. However, our result showing that glial overexpression of dPC^{+} does not inhibit 1 hr memory in a $DC0^{B3}$/+ background may be interpreted in two different ways. We propose that the reduction in endogenous dPC in a $DC0^{B3}$/+ background reduces the total amount of dPC in this line, decreasing overexpression and preventing memory loss. On the other hand, this result is also consistent with a model where PKA mediates dPC's effects on AMI, suggesting that a more complicated feedback interaction between PKA and dPC may regulate AMI.

Because generation of reactive oxygen species (ROSs) increases with age, and excess ROSs disrupt cellular functions, oxidative stress has been widely accepted as the predominant cause of both organismal aging and AMI (Balaban et al., 2005; Bishop et al., 2010). Consistent with this hypothesis, genetic and pharmacological interventions that reduce oxidative stress extend lifespan and improve memory in old animals (Liu et al., 2003; Sampayo et al., 2003; Sun and Tower, 1999). Since PC is a mitochondrial anapleurotic enzyme, we first hypothesized that aging-dependent increases in dPC amounts might be associated with increased ROS production. However, our data show that increased oxidative stress does not elevate dPC amounts, and increased dPC amounts do not increase oxidative stress. Furthermore, memory defects caused by increased oxidative stress do not correspond to aging-induced memory defects and decreased dPC activity does not protect against memory defects due to increased oxidative stress. Thus, we propose that dPC-dependent AMI is unlikely to be associated with increased oxidative stress.

Rather than increasing oxidative stress, we demonstrate that dPC causes AMI by inhibiting production of D-serine, a coagonist of NMDARs. D-serine has been reported to be the preferential NMDAR coagonist at synapses (Mothet et al., 2000) and is secreted by glia upon glutamate stimulation (Bains and Oliet, 2007; Schell et al., 1995). Furthermore, degradation of D-serine has been shown to protect cells from NMDA-induced neurotoxicity (Papouin et al., 2012). However, mechanisms that inhibit D-serine production or secretion have not been well characterized. Our results suggest that an inhibitory feedback mechanism exists where neuronal activity-dependent increases in PKA activity cause increased glial dPC synthesis, leading to decreased D-serine production and subsequent dampening of neuronal activity. Thus, glial D-serine release may modulate the kinetics of NMDAR activation, enhancing early activation through glutamate-dependent release and later decreasing activation upon increases in postsynaptic PKA activity. Alternatively, PKA-dependent inhibition of D-serine release may serve as a neuroprotective mechanism. We propose that aging causes dysregulation of this pathway, leading to decreased NMDAR activity

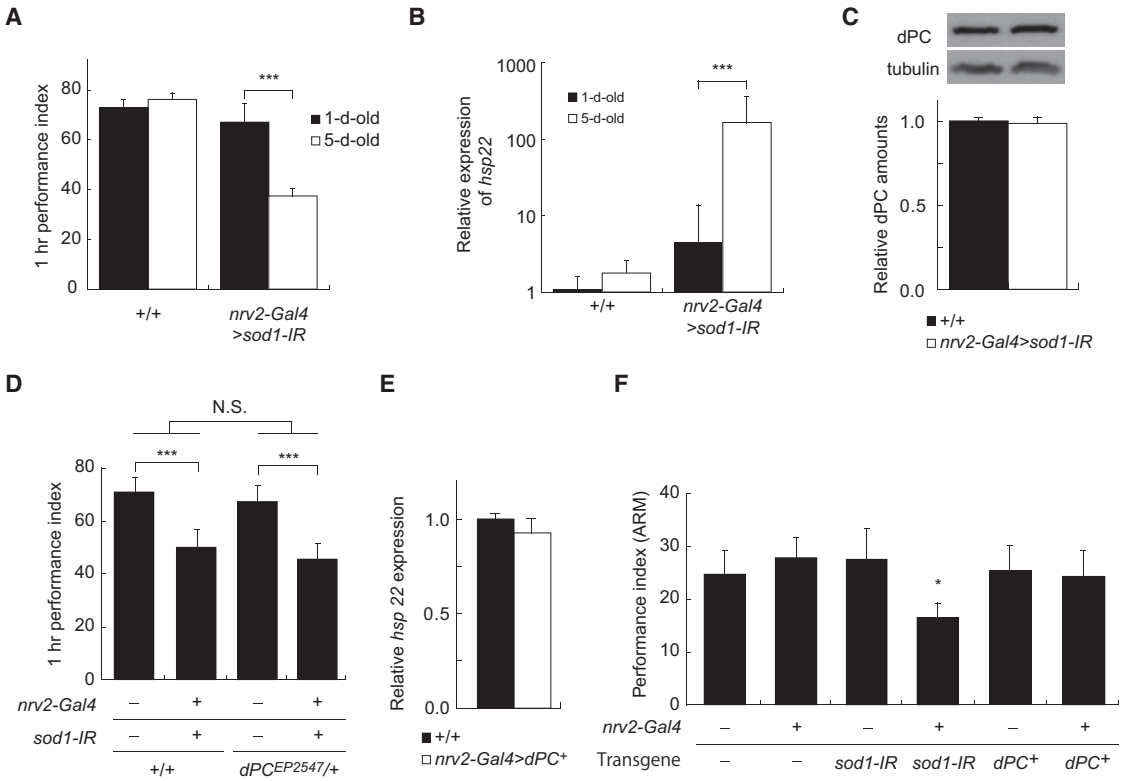

Figure 5. dPC Activity Is Not Associated with Oxidative Stress

(A) One-hour memory in *nrv2-Gal4>sod1-IR* flies is disrupted at 5 days of age. Two-way ANOVA of the data indicate significant differences due to genotype ($F_{1,34}$ = 53.96, p < 0.0001), age ($F_{1,34}$ = 25.93, p < 0.0001), and interaction between age and genotype ($F_{1,34}$ = 40.20, p < 0.0001). ***p < 0.001. n = 8–12.

(B) *sod1* knockdown in glia increases oxidative stress. A significant increase in *hsp22* expression is observed in 5-day-old *nrv2-Gal4>UAS-sod1-IR* flies. Two-way ANOVA reveals significant differences due to *sod1-IR* expression ($F_{1,12}$ = 12.23, p < 0.0044), age ($F_{1,12}$ = 11.83, p < 0.0049), and interaction between *sod1-IR* expression and age ($F_{1,12}$ = 11.72, p < 0.0051). ***p < 0.001 as determined by Bonferroni post hoc tests, n = 4.

(C) dPC amounts in 5-day-old *nrv2-Gal4>sod1-IR* flies and wild-type flies have no significant differences. p = 0.8361, determined by unpaired, two-tailed t test (t_6 = 0.2160). n = 4.

(D) The dPC^{EP2547}/+ mutation does not ameliorate memory defects in 5-day-old *nrv2-Gal4>sod1-IR* flies. Significant differences due to *sod1-IR* expression ($F_{1,32}$ = 110.1, p < 0.0001) were identified by two-way ANOVA, but no differences due to genetic background ($F_{1,32}$ = 0.2778, p = 0.6018) or interaction between background and *sod1-IR* expression were detected ($F_{1,32}$ = 0.1860, p = 0.6692). ***p < 0.001. n = 8–10.

(E) Overexpressing dPC^+ in glia (*nrv2-Gal4 > UAS-dPC*$^+$) does not increase *hsp22* expression, as assayed by unpaired, two-tailed t test (t_6 = 0.8069, p = 0.4505).

(F) ARM is disrupted by oxidative stress but not by overexpressing dPC^+ in glia. One-way ANOVA demonstrated significant differences ($F_{5,78}$ = 2.802, p = 0.0222). *p < 0.05, Bonferroni post hoc tests, compared to driver and transgene alone controls.

(Figure 6E). Although the cause of this dysregulation is still unknown, it seems to occur independently of oxidative stress and PKA activity.

NMDARs are required for multiple learning and memory phases, but moderate reductions in NMDAR expression using an RNAi-based strategy have shown that two phases, MTM and long-term memory (LTM), are particularly sensitive to decreases in NMDAR activity (Wu et al., 2007). MTM and LTM are the two memory phases predominantly affected by AMI (Mery, 2007; Tamura et al., 2003), supporting a model where AMI is caused by mild inhibition of NMDAR activity.

Amounts of D-serine have been reported to decrease upon aging in the rat hippocampus, a result consistent with our data from flies. Further, an age-related decline in hippocampal synaptic plasticity is ameliorated by exogenously applied D-serine (Junjaud et al., 2006). These observations suggest that a PC-mediated, oxidative stress-independent signaling pathway may also play a role in mammalian AMI.

EXPERIMENTAL PROCEDURES

Fly Stocks and Drug Treatments

All *Drosophila melanogaster* lines used in this study were outcrossed to our wild-type line, *w*(CS10) (Dura et al., 1993), for at least six generations. Mutations not linked to a miniwhite marker were identified by PCR at each generation. *UAS-DC0*$^+$ *F5.9*, described previously (Kiger et al., 1999; Kiger and O'Shea, 2001), was used as *UAS-DC0*$^+$. To generate *UAS-dPC*$^+$ flies, we subcloned the *CG1516* coding region into pUAST, a *Drosophila* expression vector, and injected with pUChspΔ2-3 into w(CS10) embryos. $CG1516^{EP2547}$ (dPC^{EP2547}) and $CG1516^{CB0292-3}$ ($dPC^{CB0292-3}$) flies were obtained from the Szeged *Drosophila* stock center, *UAS-sod1-IR* flies were obtained from the Vienna *Drosophila* RNAi Center, and *UAS-mito-GFP* (*P{UAS-mito-HA-GFP.AP}3*) and $CG1516^{MI02451}$ ($dPC^{MI02451}$) flies (Venken et al., 2011) were obtained from the Bloomington *Drosophila* stock center. The glial GeneSwitch

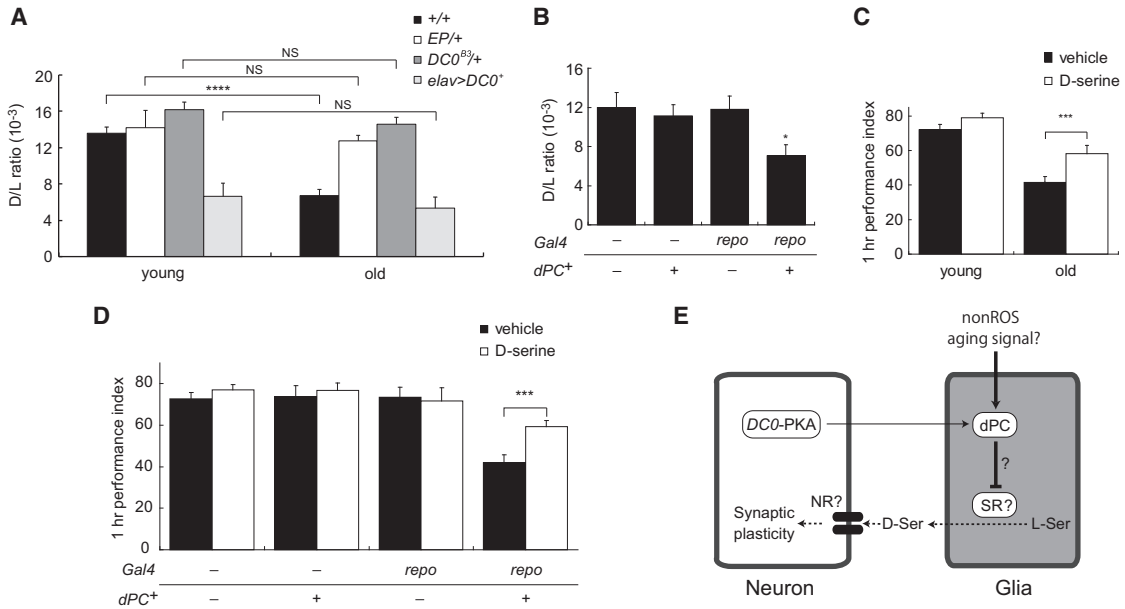

Figure 6. Reduced Amounts of D-serine Are Associated with Increased dPC Activity and AMI

(A) The D-serine-to-L-serine ratio in young (3- to 5-day-old) and old (20- to 25-day-old) wild-type, *dPC*^{EP2547}/+ (*EP*/+), *DC0*^{B3}/+, and *elav-Gal4>UAS-DC0*$^{+}$ flies. Significant differences due to genotype ($F_{3,42}$ = 30.09, p < 0.0001), age ($F_{1,42}$ = 13.70, p = 0.0006), and interaction between age and genotype ($F_{3,42}$ = 3.219, p = 0.0322) were identified by two-way ANOVA. ****p < 0.0001.

(B) Increased expression of *dPC*$^{+}$ in glia decreases amounts of D-serine. One-way ANOVA demonstrated significant differences due to genotype ($F_{3,20}$ = 5.548, p = 0.0062). *p < 0.05 compared to all other genotypes.

(C) D-serine feeding improves 1 hr memory in 25-day-old but not young (3- to 5-day-old) wild-type flies. Two-way ANOVA identified significant differences due to D-serine feeding ($F_{1,28}$ = 17.18, p = 0.0003), age ($F_{1,28}$ = 90.46, p < 0.0001), and interaction between D-serine feeding and age ($F_{1,28}$ = 6.655, p = 0.0154). ***p < 0.001.

(D) D-serine feeding improves 1 hr memory in transgenic flies overexpressing *dPC*$^{+}$ in glia. Significant differences due to genotype ($F_{3,52}$ = 26.70, p < 0.0001), D-serine feeding ($F_{1,52}$ = 5.766, p = 0.0199), and interaction between genotype and feeding ($F_{3,52}$ = 4.339, p = 0.0084) were identified by two-way ANOVA. ***p < 0.001. n = 6–12 for all behavioral data and n = 4–6 for biochemical data.

(E) A working model for AMI. dPC activity in glia is regulated by PKA activity in neurons and an as-yet-unidentified oxidative stress-independent aging signal. Increases in dPC activity lead to decreased production of D-serine, possibly through inhibition of serine racemase. We propose that reductions in D-serine may decrease NMDA-type glutamate receptor activity leading to decreased synaptic plasticity and AMI. In the model, thick lines represent pathways upregulated upon aging, dotted lines represent pathways downregulated upon aging, and thin lines represent pathways unaffected by aging. Question marks placed after the oxidative-stress-independent aging signal and between dPC and SR indicate that these pathways have not been clearly identified. The question marks after NMDAR and SR indicate that NMDAR has not yet been shown to affect AMI in flies and that it is not clear whether neuronal or glial SR is responsible for decreases in D-serine upon aging.

GAL4 driver *GSG3285-1* (Nicholson et al., 2008) was a gift from H. Keshishian (Yale University). Fly stocks were maintained at 25°C ± 2°C and 60% ± 10% relative humidity under a 12 hr/12 hr light/dark cycle. Approximately 50 flies (for behavior tests) or 20 flies (for lifespan tests) were raised in food vials and transferred to fresh vials every 2 or 3 days.

For paraquat (PQ, Sigma), RU486 (RU, Sigma), and D-serine (Wako) treatment studies, approximately 100 flies were transferred into food vials containing indicated concentrations of each drug for indicated times.

Learning and Memory Assays

Standard single-cycle olfactory conditioning was performed as previously described (Tully and Quinn, 1985) and is described in more detail in the Supplemental Information. Briefly, flies are trained by exposing them to two odors, one of which is paired with aversive electrical shocks. Learning and memory of this association can be tested at various times after training by allowing flies to choose between the two odors. Learning and memory retention are quantified as a performance index calculated by subtracting the percentage of flies that chose the shock-paired odor from the percentage that chose the unpaired odor. One sample (n) in a performance score consists of the average for two experiments where the CS+ and the CS− are alternated. Peripheral controls for memory experiments, including odor acuity and shock reactivity assays, were performed on experimental and control flies to determine that sensitivity to odors and shock were unaffected in our mutants.

Anesthesia-resistant memory (ARM) was assayed as 3 hr memory where a cold shock was given to flies 2 hr after training (1 hr before testing), by placing vials containing trained flies into ice water for 90 s. Cold shock erases anesthesia-sensitive forms of memory (short-term memory and middle-term memory) leaving only ARM.

Generation of anti-PC Antibodies

We generated two antibodies to dPC in this study. α-dPC-y was raised against the dPC C-terminal amino acid sequence, PHGGFPEPLRSRC, and α-dPC-m was raised against a 16 residue peptide, PQTPLATTLKPALVSPH, corresponding to residues 514 to 530 of dPC. Peptides were fused to keyhole limpet hemocyanin, and antibodies were generated using previously described protocols (Saitoe et al., 1997). Anti-dPC antibodies were affinity purified from serum using antigen-peptide-coupled columns (EZ Antibody Production and Purification Kit, Carboxyl Reactive, Pierce). Both antibodies recognize dPC by western and were used at a 1:1,000 dilution. For immunostaining, α-dPC-m was used at a 1:500 dilution.

Quantification of Transcripts

Transcript levels were quantified using real-time PCR (Applied Biosystems, model 7500) as described previously (Yamazaki et al., 2007). Fly heads were harvested on dry ice, and total RNA was isolated using TRIzol reagent (Invitrogen). cDNA was synthesized using a High-Capacity cDNA Archive Kit (Applied Biosystems), and qPCR was performed using SYBR Green-based chemistry using specific primers. Expression of each transcript was normalized to that of *GAPDH2*.

Identification of dPC-Positive Cells

For dPC localization studies, *Repo-GAL4>UAS-mito*-GFP flies with proboscides removed were fixed in 4% paraformaldehyde for 2 hr at 4°C. Flies were then washed in PBS and brains were dissected. Brains were autoclaved for 10 min at 120°C (Hofman and Taylor, 2013) in 10 mM NaCitrate (pH 6.0), washed 3× in PBS with 0.2% Triton X-100 (PBST), and blocked in PBST containing 5% fetal bovine serum. They were then incubated overnight at 4°C with primary antibodies, 1:1,000 chick-anti-GFP antibody (MBL) and 1:500 anti-pyruvate carboxylase antibody in PBST containing 5% fetal bovine serum. After washing (3× PBST), brains were incubated overnight at 4°C with secondary antibodies, 1:1,000 Alexa 488-conjugated anti-chick IgG, and 1:1,000 Alexa 555-conjugated anti-rabbit IgG. Brains were mounted and observed using a confocal microscope (LSM-710, Zeiss).

dPC Activity Assays

dPC activity was measured by coupling the PC reaction with an MDH reaction as described previously (Modak and Kelly, 1995). To measure the dPC-myc activity in a cell-free system, we employed a standard immunoprecipitation protocol using anti-myc antibody to purify dPC-myc from head extracts of transgenic flies. Immunoprecipitates were resuspended in complete assay mixture (0.1 M Tris-HCl [pH 8.0], 0.1 M $NaHCO_3$, 0.1 M KCl, 5 mM $MgCl_2$, 10 mM pyruvate, 4 mM ATP, 200 μM acetyl-CoA, 10 U pig heart MDH, and 150 μM NADH) or in assay mixtures lacking one component. Reactions were initiated by the addition of pyruvate and assays were performed at 30°C for 30 min. MDH-dependent NADH consumption, monitored by measuring absorbance at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$), was determined as an indicator of PC activity.

To measure endogenous dPC activity, we prepared mitochondrial fractions from fly head extracts (Walker et al., 2006) because PC has been reported to function in mitochondria (Hertz et al., 2007; Shank et al., 1985). Frozen fly heads were gently crushed in mitochondrial isolation medium (350 mM sucrose, 20 mM Tris-HCl [pH 7.5], and 0.15 mM $MgCl_2$) and centrifuged at 1,000 × *g* for 5 min at 4°C. Supernatants were then centrifuged at 13,000 × *g* for 5 min at 4°C. Mitochondrial pellets were resuspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 150 mM KCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, and 1× protease inhibitor cocktail [Roche Applied Science]). We assayed 10 μg of mitochondrial protein for PC activity as described above.

D- and L-Serine Measurements

Fly brains were dissected from heads in chilled Ca^{2+}-free HL-3 solution on ice. After briefly rinsing with ice-cold Ca^{2+}-free HL-3 solution (Stewart et al., 1994) (5 mM HEPES [pH 7.3], 70 mM NaCl, 5 mM KCl, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 115 mM sucrose, 5 mM trehalose, 15 μg/ml phenol red), ten brains were homogenized with 100 μl ice-cold 5% perchloroacetic acid and centrifuged. Soluble fractions were neutralized with 1 M potassium carbonate. D- and L-serine were measured by high-performance liquid chromatography (HPLC, EDT-300, Eicom) combined with fluorescence detection (Hitachi S-3350; excitation at 340 nm, emission at 440 nm) and quantified with a PowerChrom (AD Instruments) using external standards (Sigma). Precolumn derivatization was performed with 4 mM o-phthaldialdehyde/N-acetyl-L-cysteine (pH 10.0) in an autosampler (Model 231XL; Gilson) at 10°C for 5 min. Derivatives were then separated on a liquid chromatography column (EX-3ODS; φ 4.6 mm and 100 mm long; Eicom) at 30°C with 18% methanol in 0.1 M phosphate buffer (pH 6.0). Detection limits for both D-serine and L-serine were 20 fmol. D- and L-serine assays were performed blind to the genotypes and ages of the flies from which extracts were made.

Statistics

All data in graphs are expressed as means ± SEMs. Statistical analyses were performed as described in the figure legends using Prism version 5.01 (GraphPad). Bonferroni post hoc tests were performed when one-way or two-way ANOVA revealed significant differences between groups. For lifespan analyses, p values were calculated using the log rank (Mantel-Cox) methods. Unpaired, two-tailed t tests were used to identify significant differences in experiments comparing two conditions. p values < 0.05 were considered to be statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.09.039.

AUTHOR CONTRIBUTIONS

M.S. supervised the study. D.Y., J.H., and M.S. contributed to study design. D.Y., H.N., M.T., Y.Y., and T.I. performed and analyzed proteomics experiments. D.Y., J.H., and T. Miyashita performed biochemical experiments. J.H., K.U., T. Miyashita, and T. Masuda performed and analyzed immunohistochemical experiments. D.Y. and T. Miyashita generated antibodies. T.K. and Y. Honda measured and quantified brain D-serine amounts with help from K.U., J.H., and M.S. Y. Hirano, S.S., and S.N. characterized the relationship between PC and oxidative stress. T.U. and M.M. performed various molecular experiments and generated reagents used in this study. D.Y., J.H., T. Masuda, and M.S. performed behavioral experiments. D.Y., J.H., and M.S. analyzed the data. J.H. and M.S. wrote the paper.

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